REMARKS

This document is filed in reply to the non-final Office Action dated March 11, 2009 ("Office Action").

Applicants have amended claims 1 and 14 to more particularly point out the subject matter they deem as their invention. Support for amendments to claim 1 is found in original claims 10, 12, and 13; support for amendments to claim 14 is found in the Specification at page 5, lines 9-14; page 6, lines 20-25; page 7, lines 2-5; page 25, lines 6-9; page 27, lines 20-22; and in original claim 26. In particular, support for "via a coronary artery supplying a cardiac muscle in the heart" can be found at page 6, lines 22-25.[1] New claims 29-42 have been added; support for which can be found in the Specification and is summarized in the table below.

Claims	Support in the Specification
claims 29, 30, and 35	original claim 12
claim 31	page 6, lines 19-20 and page 13, lines 11-12; original claims 1, 10, and 13
claim 32	original claim 3
claim 33	original claim 4
claim 34	original claim 11
claims 36 and 37	original claim 13
claims 38 and 41	original claim 16
claims 39 and 42	original claim 17
claim 40	page 5, lines 23-24

Claims 2, 5-10, 12, 13, 15, 18-23, and 25-28 are cancelled. The cancellation of claims 13 and 23 necessitated dependency changes to claims 11 and 24; these claims have also been amended

[1] This passage of the Specification discloses that a drug can be administered using a balloon catheter. As commonly known, a balloon catheter is positioned by a cardiologist into a coronary artery during cardiac catheterization. See a page from Wikipedia entitled "Balloon Catheter" (Exhibit A). Thus, administering a drug using a balloon catheter is "via a coronary artery supplying a cardiac muscle in the heart." In other words, the Specification sufficiently supports the newly added limitation.

Note that the exact language in amended claim 14 does not have to be set forth verbatim in the Specification. In *In re Wright*, 9 USPQ2d 1649 (Fed. Cir. 1989), the Federal Circuit, in reversing a Board's 35 U.S.C. § 112, first paragraph rejection, held that there was adequate written description support for applicant's claim limitation, despite the fact that it was not set forth "*in haec verba*" (i.e., "in these words" or "verbatim") in the Specification.

to promote clarity. Typographical errors were corrected in claims 3, 14, and 16. No new matter has been introduced.

Upon entry of the proposed amendments, claims 1, 3, 4, 11, 14, 16, 17, 24, and 29-42 will be pending and under examination. Applicants respectfully request that the Examiner reconsider this application in view of the following remarks.

Objection to claim 14

Claim 14 is objected to because the term "tachycardia" is misspelled. See the Office Action, page 2, lines 8-9. This typographical error has been corrected.

Rejection under 35 U.S.C. § 112, first paragraph (Written Description)

The Examiner rejects claims 14-25 and 27 for failing to meet the Written Description requirement. See the Office Action, page 2, lines 16-19. Claims 15, 18-23, 25, and 27 are cancelled and, consequently, only claims 14, 16, 17, and 24 are subject to this rejection. Applicants will address this rejection in view of amended claim 14.

Amended claim 14, from which the other rejected claims depend, is drawn to a method for treating a heart disease characterized by hypertrophy, arrhythmia, or tachycardia. The method includes administering directly to a cardiac muscle or via a coronary artery supplying a cardiac muscle an expression vector containing a sequence encoding a CD9 protein whereby the heart disease is treated by suppressing cardiac hypertrophy or cardiac tachycardia.

The Examiner states that the instant claims cover use of (1) "any expression inducing substance for expressing an endogenous CD9 gene (see at least dependent claim 27)" and (2) "a gene therapy agent used for transferring a CD9 gene other than an expression vector comprising a CD9 gene (see at least dependent claims 25-26)." See the Office Action, page 3, lines 9-12; *emphases added.*

First, Applicants point out that claim 14, as amended, is not about use of "any expression inducing substance for expressing an endogenous CD9 gene." Indeed, claim 14 requires the administration of an expression vector that includes a sequence that encodes a CD9 protein. In other words, the claim is drawn to use of a substance for expressing an exogenous CD9 gene not for expressing an endogenous CD9 gene. Thus, the Examiner's first statement is inapplicable to amended claim 14. Second, claim 14, as amended, does not relate to use of

"a gene therapy agent used for transferring a CD9 gene other than an expression vector comprising a CD9 gene." Amended claim 14 covers administration of an expression vector comprising a CD9 gene. It does not require any other gene therapy agent. Again, the Examiner's second statement is inapplicable in view of the amendments to claim 14. Applicants point out that claims 25-27 are cancelled, i.e., claims specifically mentioned by the Examiner.

In view of the above amendments and remarks, Applicants submit that the Examiner's grounds for rejection have been overcome and that the Specification sufficiently conveys to a skilled artisan that they had possession of the invention of claim 14, at the time of filing. Applicants respectfully request withdrawal of the rejection of claim 14 and its dependent claims, i.e., claims 16, 17, and 24.

Applicants have proposed new claims 35-42, which depend from claim 14. These claim further limit the method of claim 14 to the treatment of particular diseases (i.e., claims 38, 39, 41, and 42) or to use of particular expression vectors that contain a CD9 gene (i.e., claims 35-37 and 40). For at least the same reasons set forth above, they also meet the Written Description requirement.

Rejection under 35 U.S.C. § 112, first paragraph (Enablement)

Claims 14-28 are rejected for lack-of-enablement. See the Office Action, page 5, line 8. Only claims 14, 16, 17, and 24 are subject to this rejection as claims 15, 18-23, and 25-28 are cancelled. Applicants will first discuss an invention that the Examiner deems enabled by the Specification.

The Examiner states that the Specification is:

> "enabling for: A method for suppressing cardiac hypertrophy or cardiac tachycardia in a subject having a heart disease, said method comprises [1] administering directly to a cardiac muscle in the heart of said subject [2] an expression vector comprising a sequence encoding a CD9 protein, wherein the heart disease is characterized by myocardial infarction, hypertrophy, arrhythmia or tachycardia, and [3] wherein cardiac hypertrophy or cardiac tachycardia is suppressed in said subject[.]"

See the Office Action, page 5, lines 9-15; *emphases added.* In contrast, the Examiner states that the Specification is not enabling for a "method of treating a heart disease characterized by

myocardial infarction, hypertrophy, arrhythmia or tachycardia by simply expressing a CD9 gene in the heart of a subject in need as broadly claimed [in previously presented claim 14]." See the Office Action, page 5, lines 16-18; *emphases added.* Applicants notice that the invention that the Examiner deems enabled and the method of previously presented claim 14 differ in that the former recites three additional limitations, i.e., the three above-itemized limitations. It seems that the Examiner considers that the three above-itemized limitations are essential for a skilled artisan to make and use the invention.

In an effort to place this application in condition for allowance, Applicants have amended claim 14 to include the three above-itemized limitations. Amended claim 14 now covers a method of (1) administering directly to a cardiac muscle or via a coronary artery supplying a cardiac muscle (2) an expression vector containing a sequence encoding a CD9 protein for treating a heart disease characterized by hypertrophy, arrhythmia, or tachycardia and (3) wherein the heart disease is treated by suppressing cardiac hypertrophy or tachycardia.

Applicants now address two differences between the method of amended claim 14 and the invention deemed enabled by the Examiner. First, amended claim 14 covers "a method for treating a heart disease ... wherein the heart disease is treated by suppressing cardiac hypertrophy or cardiac tachycardia" whereas the invention deemed enabled covers "a method for suppressing cardiac hypertrophy or cardiac tachycardia in a subject having a heart disease." Although their word orders are different, the mechanism/outcome of two methods is the same, i.e., suppression of cardiac hypertrophy or cardiac tachycardia. Second, the invention deemed enabled covers a method of administering a drug "directly to a cardiac muscle," whereas amended claim 14 covers a method of administering a drug "directly to or via a coronary artery supplying a cardiac muscle." Applicants submit that the two administration routes recited in claim 14 are essentially the same. It commonly known that only coronary arteries supply the cardiac muscles. See a Wikipedia page entitled "Coronary Circulation" (Exhibit B). As a drug administered via a coronary artery would only go to a cardiac muscle, the two administration routes recited in amended claim 14 are essentially "directly to a cardiac muscle." In sum, the two differences between the two methods in these aspects are insignificant. For the foregoing reasons, Applicants submit that the Specification is enabling for amended claim 14 for the same reasons that it is for the invention deemed enabled by the Examiner.

Applicants now turn to the other rejected claims. Claims 16 and 17 further limit the types of heart diseases treated by the method of claim 14, i.e., ischemic heart disease and myocardial infarction, respectively. Claim 24 recites molecular causes for the cardiac hypertrophy or cardiac tachycardia, which is suppressed by the method of claim 14. The additional limitations recited in claims 16, 17, or 24 do not involve active steps that are alone relevant to an enablement rejection. Thus, the Speciation is enabling for claims 16, 17, and 24 for the reasons that it is for claim 14. Applicants respectfully request withdrawal of the lack-of-enablement rejection of claims 14, 16, 17, and 24.

In addition, Applicants have proposed new claims 35-42 which depend from claim 14. As discussed above, these claims further limit the method of claim 14 to the treatment of particular diseases (i.e., claims 38, 39, 41, and 42) or to use of particular expression vectors (i.e., claims 35-37 and 40). Since the new claims depend from amended claim 14, they also include the three above-itemized limitations. Therefore, Applicants submit that new claims 35-42 are enabled for at least the same reasons that independent claim 14 is enabled.

Rejection under 35 U.S.C. § 112, second paragraph (Indefiniteness)

Claims 2-3, 5-6, 10, and 23 are rejected for indefiniteness. See the Office Action, page 11, lines 7-9. Claims 2, 5-6, 10, and 23 are cancelled; thus, only claim 3 is at issue.

The Examiner rejects claim 3 as the term "the heart disease" lacks an antecedent basis. See the Office Action, page 11, lines 11-12. Applicants have amended claim 1, from which claim 3 depends, to recite "a heart disease." This amendment renders this rejection moot.

Rejections under 35 U.S.C. § 102(b)

Claims 1-18, 23-26, and 28 are rejected for anticipation on one or both of two grounds. Applicants will discuss each ground in turn.

I

Claims 1-13 are rejected for anticipation by Miyake *et al. Oncogene* 19:5221-5226, 2000 ("Miyake"). See the Office Action, page 12, lines 7-8. Claims 2, 5-10, and 12-13 have been cancelled. Applicants will first address this rejection in view of amended claim 1.

Amended claim 1 is drawn to a drug comprising an expression vector containing a CD9 gene. The expression vector can be a viral vector, i.e., an adeno-associated virus, a retrovirus, a

herpesvirus, a herpes simplex virus, a lentivirus, a Sendai virus, a poxvirus, a poliovirus, a symbis virus, or a vaccinia virus.

According to the Examiner, Miyake describes, "a composition comprising a replication-deficient adenovirus encoding ... CD9 cDNA." See the Office Action, page 12, lines 14-16; *emphasis added.* He concludes, "[t]he composition of Miyake is indistinguishable from the drug as claimed [in claim 1]." See the Office Action, page 12, line 21.

Applicants point out that amended claim 1 does not cover a drug comprising an adenovirus. In other words, the composition Miyake is distinguishable from the drug of claim 1. Thus, Miyake does not anticipate amended claim 1; nor its dependent claims, i.e., claims 3, 4, and 11. Applicants respectfully request the withdrawal of this rejection.

Applicants have added claims 29 and 30, which depend from claim 1. These claims simply specify that the expression vector of claim 1 is either a viral (claim 29) or a non-viral (claim 30) vector; these claims are not anticipated by Miyake for the reasons that claim 1 is not.

Additionally, Applicants have proposed new claim 31. This claim is also drawn to a drug comprising an expression vector containing a CD9 gene. The expression vector is an adenovirus and the expression of CD9 is promotered by a Rous sarcoma virus (RSV) promoter. Applicants note that Miyake describes a composition in which the expression of the CD9 gene is promotered by the human CMV immediate early promoter. See Miyake, page 5224, right column, para. "Recombinant adenoviruses," line 3. As the Miyake composition includes a CMV promoter and the drug of claim 31 includes an RSV promoter, the two compositions are distinguishable. Consequently, Miyake does not anticipate new claim 31. Nor does it anticipate new claims 32-34 which depend from claim 31.

II

Claims 1-18, 23-26, and 28 are rejected for anticipation by Ushikoshi *et al. Circulation,* Supplement III, 110, page 8, 2004 ("Ushikoshi"). See the Office Action, page 13, lines 1-2. The Examiner states: "Applicant[s] cannot rely upon the foreign priority papers to overcome this rejection because a translation of said papers has not been made of record in accordance with 37 CFR 1.55. See MPEP § 201.15." See the Office Action, page 13, lines 11-13.

Applicants submit herewith a copy of the English translation (Exhibit C) of the foreign priority document, i.e., Japanese Application No. 2003-432279, filed December 23, 2003, along

with a Verification of Translation (Exhibit D) by Mr. Akira Nishio, who translated Japanese into English. The Verification of Translation declares that the English translation is both true and complete. In accordance with MPEP § 201.15, Applicants have satisfied the requirements necessary to rely on the above-mentioned priority document to overcome the rejection.

Applicants point out the foreign priority document, filed December 23, 2003, antedates Ushikoshi, which was published on October 26, 2004. Consequently, Ushikoshi does not qualify as prior art and cannot be relied upon for this rejection. Thus, Applicants respectfully request the rejection's withdrawal.

CONCLUSION

It is believed that all of the pending claims have been addressed. However, the absence of a reply to a specific rejection, issue or comment does not signify agreement with or concession of that rejection, issue or comment. In addition, because the arguments made above may not be exhaustive, there may be reasons for patentability of any or all pending claims (or other claims) that have not been expressed. Finally, nothing in this paper should be construed as an intent to concede any issue with regard to any claim, except as specifically stated in this paper, and the amendment of any claim does not necessarily signify concession of unpatentability of the claim prior to its amendment.

The Petition for Extension of Time fee in the amount of $245 is being paid concurrently herewith on the Electronic Filing System (EFS) by way of Deposit Account authorization. Please apply any other charges or credits to Deposit Account No. 50-4189, referencing Attorney Docket No. 55801-003US1.

Respectfully submitted,

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Exhibit A

Balloon catheter

From Wikipedia, the free encyclopedia

A **balloon catheter** is a type of "soft" catheter with an inflatable "balloon" at its tip which is used during a catheterization procedure to enlarge a narrow opening or passage within the body. The deflated balloon catheter is positioned, then inflated to perform the necessary procedure, and deflated again in order to be removed.

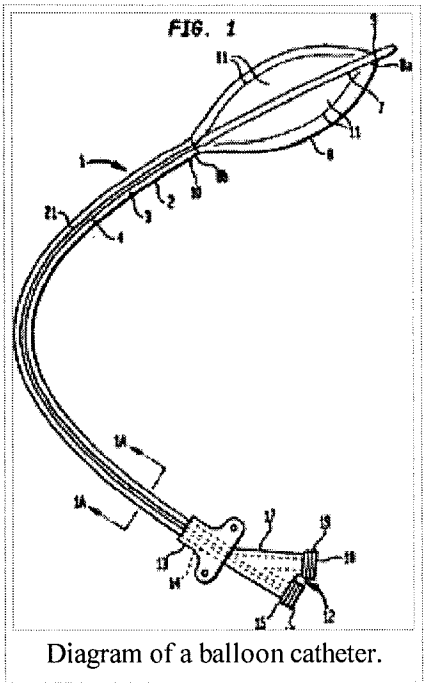


Diagram of a balloon catheter.

Some common uses include:

- angioplasty or balloon septostomy, via cardiac catheterization (**heart cath**)
- tuboplasty via uterine catheterization

Angioplasty balloon catheters

Balloon catheters used in angioplasty are either of Over-the-Wire(OTW) or Rapid Exchange(Rx) design. When a balloon catheter is used to compress plaque within a clogged coronary artery it is referred to as a plain old balloon angioplasty or POBA. Balloon catheters are also utilized in the deployment of stents during angioplasty. Balloon catheters are supplied to the cath lab with a stent premounted on the balloon. When the cardiologist inflates the balloon it expands the stent. When the cardiologist subsequently deflates the balloon the stent stays behind in the artery and the balloon catheter can be removed. Stents that are used in conjunction with a balloon catheter are known as balloon expandable stents.

External links

- Video of how the original balloon embolectomy catheter works (http://web.mit.edu/invent/www/ima/fogarty_video.html) . From MIT Press.

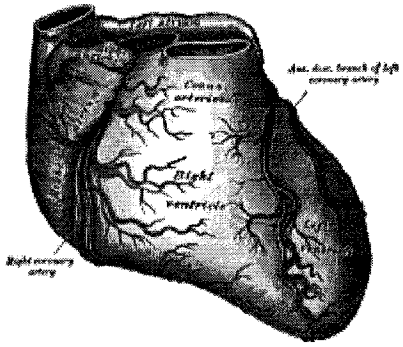
Retrieved from "http://en.wikipedia.org/wiki/Balloon_catheter"
Categories: Catheters | Medical equipment stubs

Exhibit B

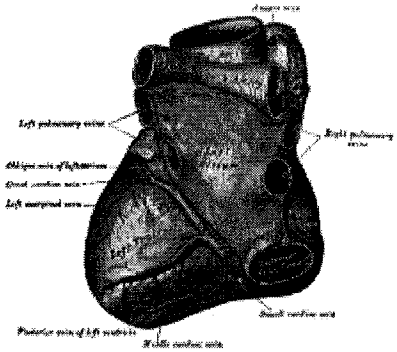
Coronary circulation

From Wikipedia, the free encyclopedia

Coronary circulation



An anterior view of the heart shows the right coronary artery and the anterior descending branch of the left coronary artery.



Base and diaphragmatic surface of heart.

MH *Coronary+Vessels (http://www.nlm.nih.gov/cgi/mesh/2007/MB_cgi?mode=&term=Coronary+Vessels)*

Coronary circulation is the circulation of blood in the blood vessels of the heart muscle. Although blood fills the chambers of the heart, the muscle tissue of the heart (the myocardium) is so thick that it requires coronary blood vessels to deliver blood deep into it. The vessels that deliver oxygen-rich blood to the myocardium are known as coronary arteries. The vessels that remove the deoxygenated blood from the heart muscle are known as coronary veins.

The coronary arteries that run on the surface of the heart are called epicardial coronary arteries. These arteries, when healthy, are capable of autoregulation to maintain coronary blood flow at levels appropriate to the needs of the heart muscle. These relatively narrow vessels are commonly affected by atherosclerosis and can become blocked, causing angina or a heart attack. (See also: circulatory system.) The coronary arteries that run deep within the myocardium are referred to as subendocardial.

The coronary arteries are classified as "end circulation", since they represent the only source of blood supply to the myocardium: there is very little redundant blood supply, which is why blockage of these vessels can be so critical.

Exhibit C

[Document]CLAIMS

1. A drug for preventing or treating a heart disease, comprising an expression vector containing a CD9 gene as the active ingredient.

2. The drug for preventing or treating a heart disease according to Claim 1, wherein the heart disease is associated with cardiac hypertrophy and/or tachycardia.

3. The drug for preventing or treating a heart disease according to Claim 2, wherein cardiac hypertrophy or tachycardia is caused by at least one of HB-EGF (heparin binding epidermal growth factor), HGF (hepatocyte growth factor) or angiotensin 2.

4. The drug for preventing or treating a heart disease according to any of Claims 1 to 3, wherein the expression vector is a viral vector or a non-viral vector.

5. The drug for preventing or treating a heart disease according to Claim 4, wherein the viral vector is adenovirus, adeno-associated virus, retrovirus, herpesvirus, herpes simplex virus, lentivirus, Sendai virus, poxvirus, poliovirus, symbis virus or vaccinia virus.

6. A method for preventing or treating a heart disease, comprising expressing a CD9 gene in heart.

7. The method for preventing or treating a heart disease according to Claim 6, wherein the heart disease is associated with cardiac hypertrophy and/or tachycardia.

8. The method for preventing or treating a heart disease according to Claim 7, wherein cardiac hypertrophy or tachycardia is caused by at least one of HB-EGF (heparin binding epidermal growth factor), HGF (hepatocyte growth factor) or angiotensin 2.

9. The method for preventing or treating a heart disease according to any of Claims 6 to 8, wherein the prevention or treatment is carried out by a gene therapy of transferring a CD9 gene.

10. The method for preventing or treating a heart disease according to Claim 9, wherein the gene therapy comprises using a drug containing an expression vector containing a CD9 gene as the active ingredient.

11. The method for preventing or treating a heart disease according to any of Claims 6 to 8, wherein the prevention or treatment comprises administration of an expression inducing substance for expressing endogenous CD9.

[Document]Specification

[Title of the invention]

DRUG FOR PREVENTING OR TREATING HEART DISEASES CONTAINING CD9 GENE

[Technical Field]

[0001]

The present invention relates to a drug for preventing or treating heart diseases, more specifically, to a drug which is used for gene therapy and prevents or treats heart diseases by transferring a CD9 gene into heart.

[Background Art]

[0002]

Heart diseases such as myocardial infarction, cardiomypathy, arrhythmia, heart failure and the like are one of three major causes for death in Japan, generating a significant problem in medical care. Conventionally, drugs such as a diuretic, β-blocker, ACE inhibitor, calcium antagonist and the like are used for treatment of heart diseases, however, these drugs do not radically treat heart diseases.

[0003]

CD9 is a membrane protein having a molecular weight of 27 kDa classified into one of transmembrane 4-times type protein super family (TM4SF). Regarding the action of CD9 in an organism, there are reports that it is a cell-specific marker derived from pre B (Masellis-Smith A et al; J Immunol. 144 15, 1607-1613, 1990), that it expresses in various cells irrespective of

hematopoietic cells or non-hematopoietic cells (Maecker H. T et al.; FASEB J. 11, 428-442, 1997, Berditchevski F; J Cell Sci. 114, 4143-4151, 2001), that it relates to aggregation and differentiation of pre B cells (Masellis-Smith A et al; J Immunol. 144, 1607-1613, 1990), that it relates to activation of platelets (Jennings LK et al.; J Biol Chem. 265, 3815-3822, 1990), that it relates to survival and adhesion of various cells typically including cancer cells (Hashida H, et al.: Br J Cancer., 89: 158-67, 2003), that it is a membrane protein indispensable for fertilization (Miyado K et al.; Science 287, 321-324, 2000), that it causes association of a plurality of proteins in a cell membrane, acts as a molecule for coordinating and promoting a mutual action between proteins, and relates to phenomena of injury healing such as cell adhesion, proliferation, differentiation, immune, hemostatis and the like (Berditchevski F; J Cell Sci. 114, 4143-4151, 2001, Klein-Soyer C et al; Arterioscler Thromb Vasc Biol. 20, 360-3).

[0004]

On the other hand, there is also a report regarding a relation between CD9 and HB-EGF (heparin binding epidermal growth factor). For example, there are reports on formation of a complex with HB-EGF or integrin α3β1 (Nakamura Y, et al: J Histochem Cytochem 49: 439-444, 2001), regulation of an activity as a juxtacrine factor of proHB-EGF as a precursor of HB-EGF (Higashiyama S., et al,: J. Cell Biol. 128, 929-938. 1995, Iwamoto R, Mekada E: Cytokine & Growth Factor Reviews. 11:

335-344, 2000), and possibility of representation of variety of an activity of HB-EGF by relating to a process of processing of HB-EGF (Nakagawa T et al; J Biol Chem. 271, 30858-30863, 1996, Nakamura K et al; J Biol Chem. 275, 18284-18290, 2000). There are also reports that kidney epithelial cells also are improved in survival by co-expression of HB-EGF and CD9 in an ischemic disorder model (Takamura T et al; Kidney Int. 55, 71-81, 1999), that, in a process of arteriosclerosis, HB-EGF is expressed in normal aorta (Miyagawa J et al; J Clin Invest. 95, 404-411, 1995) and coronary (Nakata A et al; Circulation 94, 2778-2786, 1996), on the other and, CD9 expresses in arteriosclerosis lesion and some inner membrane smooth muscle cells, and co-expression of CD-9 and proHB-EGF promotes proliferation of smooth muscle cells by proHB-EGF (Nishida M et al; Arterioscler Thromb Vasc Biol. 20, 1236-1243, 2000), thus, CD9 is possibly an important molecule for regulating an activity of HB-GF assisting a balance of proliferation and transformation of fibroblasts in a process of arteriosclerosis and tissue repair (Kirkland G et al; J Am Soc Nephrol. 9, 1464-73, 1998). Further, the present inventors have a finding that excess expression of HB-EGF in a myocardial infarction model animal promotes compensatory hypertrophy of a myocardial cell, while promotes proliferation of myofibroblasts and enhances fibering, thereby, promoting decrease in a cardiac function, that is, HB-EGF is a central factor playing an important role in progressing of pathology.

As described above, there are a lot of reports on the

action of CD9 and a relation of CD9 and HB-EGF, however, there is no report on a fact that CD9 suppresses cardiac hypertrophy, or suppresses tachycardia.

【Not patent document 1】 Nakamura Y, et al : J Histochem Cytochem 49: 439-444, 2001

【Not patent document 2】 Higashiyama S., et al,:J.Cell Biol.128,929-938.1995

【Not patent document 3】 Iwamoto R, Mekada E : Cytokine & Growth Factor Reviews. 11: 335-344, 2000

【Not patent document 4】 Nakagawa T et al; J Biol Chem. 271, 30858-30863, 1996

【Not patent document 5】 Nakamura K et al; J Biol Chem. 275, 18284-18290,2000

【Not patent document 6】 Takemura T et al; Kidney Int. 55, 71-81, 1999

【Not patent document 7】 Miyagawa J et al; J Clin Invest. 95,404-411, 1995

【Not patent document 8】 Nakata A et al; Circulation 94,2778-2786, 1996

【Not patent document 9】 Nishida M et al;Arterioscler Thromb Vasc Biol.20, 1236-1243,2000

【Not patent document 10】 Kirkland G et al; J Am Soc Nephrol. 9, 1464-73, 1998

[DISCLOSURE OF THE INVENTION]

[Problem to be solved by the invention]

[0005]

The present invention has an object of providing a drug which radically prevents or treats heart diseases. Another object is to provide a method for radically preventing or

treating heart diseases.

[Means for solving problem]

[0006]

The inventors have accomplished the present invention based on the above acknowledge

The present invention relates to a drug for preventing or treating heart diseases, containing an expression vector containing a CD9 gene as the active ingredient. In the present invention, the heart disease is associated with cardiac hypertrophy and/or tachycardia, and cardiac hypertrophy or tachycardia is caused by at least one of HB-EGF (heparin binding epidermal growth factor), HGF (hepatocyte growth factor) or angiotensin 2.

[0007]

In the above-mentioned present invention, the expression vector is a viral vector or a non-viral vector.

The present invention also relates to a method for preventing or treating heart diseases, containing expressing a CD9 gene in heart.

[0008]

In this invention, the prevention or treatment may be carried out by a gene therapy of transferring a CD9 gene, or administration of an expression inducing substance for expressing endogenous CD9. Further, the gene therapy may use a drug containing an expression vector containing a CD9 gene as the active ingredient.

[Effect of the invention]

[0009]

The drug of the present invention is used for gene therapy, and can prevent or treat radically heart diseases, particularly, heart diseases associated with cardiac hypertrophy and tachycardia by transferring a CD9 gene into heart. The prevention or treatment method for the present invention can radically prevent or treat heart diseases by allowing a CD9 gene to express in heart.

[Best mode for carrying out the invention]

[0010]

The CD9 gene used in the drug of the present invention means a gene capable of expressing CD9. The CD9 gene may be a gene in which its gene sequence is partially deleted, substituted, inserted or, other base is added, providing a polypeptide to be expressed has substantially the same effect as CD9. As the CD9 gene, a human CD9 gene described in Strausberg R. L. et al; Proc. Natl. Acd. Sci. U.S.A. 99, (26) 16899-16903, 2002 is exemplified (Gene Bank, accession No. is AAH11988, messenger RNA No. is BC011988).

[0011]

The expression vector containing a CD9 gene includes virus vector, non-virus vector, plasmid and the like. Examples of the virus vector include adenovirus, adeno-associated virus, retrovirus, herpesvirus, herpes simplex virus, lentivirus, Sendai virus, poxvirus, poliovirus, symbis virus, vaccinia

virus and the like. Examples of the non-virus vector include cationic liposome, membrane fusing liposome, cationic polymer and the like. The liposome is a capsule composed of phospholipid having a particle size of several 10 to several 100 nm, and a plasmid containing a CD9 gene in it can be filled in this capsule.

The expression vector containing a CD9 gene can be produced by using conventional gene engineering technologies, cell culture technologies and virus technologies (e.g., "Current Protocols in Molecular Biology, F. Ausubel et al. ed., (1994), John Wiley & Sons, Inc.", "Molecular Cloning (A Laboratory Manual), Third Edition. Volume 1-3. Josseph Sambrook & David W. Russel ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, New York) 2001", "Culture of Animal Cells; A Manual of Basic Technique, R. Freshney ed., 2nd edition (1987), Willey-Liss", "Frank K. Greham ed., Manipulation of adenovirus vectors, Chapter 11. p 109-p 128", "E.J. Murray ed., Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols (1991) ", "Chen, S-H, et al., Combination gene therapy for liver metastases of colon carcinoma in vivo. Proc. Natl. Acad. Sci. USA. (1995) 92, 2477-2581", and the like).

[0012]

In the drug of the present invention, an expression vector containing a CD9 gene as the active ingredient and pharmaceutically acceptable auxiliaries such as excipient, carrier, solvent and the like are mixed, and the drug can be used in the form of various preparations such as injection and

the like. The administration form of the drug of the present invention is not particularly restricted, and for example, injection, catheter, balloon catheter and the like can be adopted. In the case of an expression inducing substance for expressing endogenous CD9, systemic intravenous administration, or oral administration and the like can be adopted.

[0013]

The amount of a CD9 gene dosed by the drug of the present invention can be appropriately controlled in view of pathological condition, age, body weight and the like of a person to be receiving dosage, and when adenovirus is used as a virus vector in a clinical test of a conventional gene therapy for a patient suffering from a heart disease, safety has been confirmed using 2×10^{10} in terms of virus infectiosity titer pfu (plaque forming unit), therefore, this amount is a rough standard for the dosage. When liposome is used as a non-virus vector, a clinical test of a gene therapy for a person suffering from a heart disease has been conducted safely with a DNA amount of 2 mg, therefore, this amount is a rough standard for the dosage.

[0014]

The drug of the present invention is used in a gene therapy, and can prevent or treat heart disease of myocardial infarction, cardiomyopathy, Arrhythmia, heart failure and the like. ,especially be suitably used for heart diseases associated with cardiac hypertrophy or tachycardia.

Heart diseases can be radically treated by inhibiting pathological hypertrophy of a myocardial cell and pathological frequent pulse of a myocardial cell (tachycardia) by expression of HB-EGF, further, pathological myocardial cell enlargement or pathological myocardial cell tachycardia (tachycardia) by expression of HGF (hepatocyte growth factor) or angiotensin 2, and normalizing the function of myocardial cells. The drug of the present invention can be used for acute heart diseases since it performs an effect in several hours after administration.

In a gene therapy, a therapeutic effect for a long period of time is expected by gene expression for a long period of time, thus, the drug can be used prophylactically for preventing cardiac hypertrophy and tachycardia associated with chronic diseases such as hypertension, chronic myocardial infarction and the like.

[0015]

By allowing CD9 to express in a body, heart diseases can be prevented or treated, and the prevention and treatment of heart diseases can be carried out by a gene therapy using the drug of the present invention, and also carried out by administering a substance inducing expression of endogenous CD9 and incuding expression of endogenous CD9 in an organism.

[Example]

[0016]

The present invention will be illustrated by the following examples. The present invention is not limited to

the examples as below.

In the examples, gene engineering technologies and cell culturing technologies handling plasmids, DNA, various enzymes, E. coli, cultured cells and the like were carried out according to methods described in the above-mentioned "Current Protocols in Molecular Biology, F. Ausubel et al. ed., (1994), John Wiley & Sons, Inc." and "Culture of Animal Cells; A Manual of Basic Technique, R. Freshney ed., 2nd edition (1987), Willey-Liss", unless otherwise stated. Unless otherwise stated, general handling of adenovirus was carried out according to methods described in the above-mentioned "Frank L. Graham ed., Manipulation of adenovirus vectors, Chapter 11. p 109-p 128" and "E.J. Murray ed., Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols (1991)", and production of adenovirus was carried out according to a method described in the above-mentioned "Chen, S-H, et al., Combination gene therapy for liver metastases of colon carcinoma in vivo. Proc. Natl. Acad. Sci. USA. (1995) 92, 2477-2581". Regarding treatment effects and phenomena, a significant difference between some groups was first analyzed by Anova assay, subsequently, an assay of an individual significant difference between each two groups was analyzed by Student t-test (asymmetrical t test between two groups). A significant difference in survival ratio was analyzed using Kaplan-Meier assay.

[0017]

Adenovirus vectors used in the examples were produced

as described below.

Plasmid pADL.1/RSV (B. Fang et al., Gene Therapy (1994), 1, 247-254) was a plasmid produced by incorporating, from upstream, a 0-455 base portion from 3' side of human type 5 adenovirus, Rous sarcoma virus long-term repeat (RSV) promoter, multi cloning site, poly A signal sequence of Bovine growth hormone, and a 3328-5788 base portion from 3' side of human type V adenovirus, into pBR322 plasmid, and offered from Shu-Hsia Chen (Mount Sinai University). pADL.1/RSV plasmid was digested with restriction enzymes Hind III and Not I and purified, to give a vector to be used for ligation. On the other hand, plasmids pRcHBEGF and pRcCD9, containing cDNA of the whole length of open reading frame of human HB-EGF and cDNA of the whole length of open reading frame of monkey CD9, respectively, in plasmid pRc/CMV (Invitrogen), were offered from Mekata Eisuke (Osaka University). From both the plasmids pRcHBEGF and pRcCD9, the whole lengths of HB-EGF cDNA and CD9 cDNA were excised, respectively, using restriction enzymes Hind III and Not I, and these were subjected to agarose gel electrophoresis, the intended DNA fragment was excised and purified to give an insert to be used for ligation. Thus treated pADL.1/RSV vector and HB-EGF cDNA, CD9 cDNA inserts were subjected to a ligation reaction with T4 DNA ligase, to obtain pADL.1/RSV-HB-EGF, and pADL.1/RSV-CD9. Further, pADL.1/RSV-HB-EGF and pADL.1/RSV-CD9 were, together with pJM17 (Microbix Biosystems Inc.) of a plasmid containing a gene other than E1 region of

human type V adenovirus, co-infected in 293 cells by a calcium phosphate method. By this, a plaque containing correct intended adenovirus produced by homologous recombination emerged 10 to 14 days after co-infection. This plaque was picked up, and correct non-proliferation type recombinant adenoviruses Ad.HB-EGF and Ad.CD9 expressing intended HB-EGF and CD9 were confirmed by immune staining using an anti-HB-EGF antibody (M-18:sc-1414, SANTA CRUZ) and an anti-CD9 antibody (ALB6, IMMUNOTECH), and the like, then, viruses were proliferated by 293 cells, and concentrated and purified by a gradient centrifugal method for cesium chloride.

A recombinant adenovirus Ad.LacZ expressing a LacZ gene of E. coli used for gene transferring was produced in the same method as descriebd above, and details of the Ad.LacZ production method are described in Proc. Natl. Acad. Sci. U.S.A. (1995) 92, 2577-2581. Ad.dE1.3 contains no incorporated CD9 and HB-EGF, thus, this is a control recombinant adenovirus not expressing these genes at all. pADL.1/RSV and pJM17 containing no incorporated CD9 and HB-EGF were co-infected in 293 cells as described above, and subjected to the same method and process to produce Ad.dE1.3. HB-EGF is described in Higashiyama, S., et al. Science 251, 936-939 (1991), and its accession No. of Gene Bank is N60278. As CD9, monkey CD9 was used, and the monkey CD9 is reported as DRAP27 (diphtheria toxin receptor-associated protein) (Mitamura T, et al: J cell Biol., 118(6): 1389-1399, 1992, Gene Bank accession No. is BAA01569), and the gene sequence

thereof is believed to be almost the same and its function is also believed to be the same as human CD9.

[0018]

(Example 1) (Expression of CD9 and the like in whole heart and myocardial cell of rat and mouse)

Each endogenous expression of CD9, HB-EGF or a group of receptors of HB-EGF (EGFR, ErbB2, ErbB3, ErbB4) in the whole heart and myocardial cells was checked using a RT-PCR method. Myocardial cells and whole heart from 8-week old adult SD rats and 1-day old neonatal BALB/C mice were enzymatically treated with collagenase type 2 (WOR: CLS 2, Funakoshi catalogue No. 45004177), and isolated. Then, 1 ml of sepazole RNA I Super (NACALAI TESQUE catalogue No. 304-86) was added to homogenize, and the homogenized mixture was separated into an aqueous phase and a phenol phase by chloroform, and the aqueous phase was precipitated with isopropanol. After centrifugal separation, the product was suspended with 70% ethanol, and further centrifugally separated, to extract RNA. 1 μg of total RNA was reverse-transcribed with Super Script II reverse transcriptase (Invitrogen), and cDNA was amplified by a PCR method.

[0019]

A sense primer (5'-CCGTGATGCTGAAGCTCTTT-3', SEQ ID No. 1) and an anti-sense primer (5'-CCAAGACTGTAGTGTGGTCAT-3', SEQ ID No. 2) (Yoshizumi M., et al.: J. Biol. Chem., 267, 9467-9469, 1992) of HB-EGF, and a sense primer (5'-AGCAAGTGCATCAAATACC-3', SEQ ID No. 3) and an anti-sense primer

(5'-AATCACCTCATCCTTGTGG-3', SEQ ID No. 4) of CD9 were synthesized by requested Hokkaido System Science Co., Ltd. A group of receptors of HB-EGF referred to Sundaresan S, et al: Endocrinology 139: 4756-4764, 1988, and a sense primer (5'-ACAACTGTGAAGTGGTCCT-3', SEQ ID No. 5) and an anti-sense primer (5'-TTCCTGTAAGTTCCGCAT-3', SEQ ID No. 6) of EGFR, a sense primer (5'-AGCTGGTGACACAGCTTA-3', SEQ ID No. 7) and an anti-sense primer (5'-TGGTTGGGACTCTTGAC-3', SEQ ID No. 8) of ErbB2, a sense primer (5'-GACCTAGACCTAGACTT-3', SEQ ID No. 9) and an anti-sense primer (5'-TCTGATGACTCTGATGC-3', SEQ ID No. 10) of ErbB3, and a sense primer (5'-CATCTACACATCCAGAACA-3', SEQ ID No. 11) and an anti-sense primer (5'-AAACATCTCAGCCGTTGCA-3', SEQ ID No. 12) of ErbB4 were synthesized by requested Hokkaido System Science Co., Ltd. As an internal control, hypoxanthine phosphoribosyltransferase (HPRT) was used.

As a positive control, HepG2 cells of lung of the above-mentioned neonatal mouse and human liver cancer cells (offered by Medical Cellular Resource Center, Institute of Development, Aging and Cancer, Tohoku University) were used. In a PCR method for HB-EGF and CD9, using Promega Taq (Promega catalogue No. M1865), a cycle of heat denaturing at 94°C for 30 seconds, annealing at 56°C for 1 minute and elongation reaction at 72°C for 1 minute was repeated 38 times, and in a PCR method for a group of receptors of HB-EGF and HPRT, TAKARA Ex Taq (TAKARA catalogue No. RR001A) were used, and the analogous

procedure was performed at an annealing temperature of 55°C. As PCR thermal cycler, TAKARA TP-400 was used.

[0020]

As shown in Fig. 1, expression of HB-EGF was observed in heart both in adult rats and neonatal mice, and particularly, expression was stronger in a cultured myocardial cell, thus, it is recognized that HB-EGF was highly expressed in a myocardial cell itself in heart. Though it is recognized that ErbB2 and ErbB4 were expressed strongly in a cultured myocardial cell both in adult rats and neonatal mice, expression of EGFR was not clear in adult rats and strong expression thereof was recognized in neonatal mice. Expression of ErbB3 was not recognized in both adult rats and neonatal mice. On the other hand, though expression of CD9 was recognized in the whole heart of adult rats, expression thereof was not clear in its cultured myocardial cell, and expression thereof was not recognized both in the whole heart and cultured myocardial cells of neonatal mice. Thus, it was clarified that ErbB2 and ErbB4 included in a group of receptors of HB-EGF are relatively highly expressed in myocardial cells, while CD9 is scarcely expressed in myocardial cells.

[0021]

(Example 2)(Confirmation of gene transfer efficiency of adenovirus vector)

Myocardial cells (Aoyama T, et al: Cardiovasc Res., 55: 787-798, 2002) isolated from a 1-day old neonatal BALB/C mouse

by collagenase type 2 (WOR: CLS 2, Funakoshi catalogue No. 45004177) were disseminated on a 4-well chamber slide (Nunc: Lab-Tek, Permanox, catalogue No. 15 77437) coated with mouse laminin (Biomedical Technologies Inc. catalogue No. BT-276) at a concentration of 5×50^5/500 μl/well, and maintained by a low glucose DMEM medium (SIGMA catalogue No. D6046) containing penicillin (100 units/ml) and streptomycin (100 μg/ml), 5% inactivated Australian fetal bovine serum, and cultured in a humidified culture vessel under 95% air -5% carbon dioxide at 37°C. Gene transferring efficiency of an adenovirus vector into the resultant primary culture myocardial cell, and expression thereof were confirmed. That is, primary culture myocardial cells were infected with Ad.LacZ at MOI (multiplicity of infection: 1 MOI = 1 plaque forming unit/cell) diluted to 300, 100, 30, 10, 3 and 0, and 48 hours after, X-gal staining was effected. As shown in Fig. 2, an excellent gene transferring efficiency of 96% at MOI 30, and 80% or more at MOI 10 were obtained. Based on this result, adenovirus was infected at MOI 10 to 30, in the following experiment, and expressed sufficiently for 24 hours, then, the medium was exchanged, further, washed with serum-free medium liquid and used. When HB-EGF, angiotensin 2 or HGF is allowed act, culturing in serum-free culture liquid was carried out for further 24 hours.

[0022]

(Example 3)(Immunohistochemical staining of myocardial cell)

The cultured myocardial cells of neonatal mice obtained

above were fixed with 4% paraformaldehyde for 10 minutes, a cell membrane was perforated with 0.05% Triton X100, and blocked with 10% skimmed milk (Snow Brand Milk Products Co., Ltd.) for 60 minutes. Thereafter, a primary antibody (mouse monoclonal antibody CD9 (clone ALB6), IMMUNOTECH catalogue No. 0117) diluted 50-fold (2 ug/ml) and a goat polyclonal antibody HB-EGF (M-18)(SANTA CRUZ catalogue No. sc-144) diluted 100-fold were reacted for 1 hour, and visualization of HB-EGF was carried out while labeling with anti-goat Alexa 568 (MOLECULAR PROBES catalogue No. A-11057) and visualization of CD9 was carried out while labeling with anti-mouse Alexa 488 (MOLECULAR PROBES catalogue No. A-11029). Nucleus staining was carried out for 5 minutes with Hoechst 33342 (MOLECULAR PROBES catalogue No. H-3570) diluted 1000-fold. F-actin was labeled with rhodamine phalloidin (MOLECULAR PROBES catalogue No. R-415) diluted 500-fold and recognized. Recording of an observed image was performed by a cofocal laser microscope (Carl Zeiss product No. LSM 510). Sufficient transferring efficiency at MOI 10 was confirmed, then, expression and localization after gene transferring into a myocardial cell by Ad.HB-EGF, Ad.CD9, and Ad.HB-EGF + Ad.CD9 were confirmed by fluorescent immunohistostaining. The results are shown in Table 3.

[0023]

As shown in Fig. 3, an expression pattern when only HB-EGF was singly gene-transferred was stained in the form of grain in a cell, and when only CD9 was singly gene-transferred,

the surface of cell membrane was stained. However, when HB-EGF and CD9 were co-expressed, change of localization patterns of the proteins was confirmed. That is, when CD9 is singly gene-transferred, an expression protein of the transferred CD9 gene localizes on a membrane which is an original expression site of endogenous CD9, however, when HB-EGF and CD9 were strongly expressed together, it was found that a CD9 protein moves into a cell together with a HB-EGF protein.

[0024]

(Example 4)(Cell area and beat number of myocardial cell)

On the myocardial primary culture cell of a neonatal mouse obtained above, CD9, HB-EGF or CD9 + HB-EGF (each at MOI 10) is strongly expressed each using Ad.CD9, Ad.HB-EGF, or Ad.CD9 + Ad.HB-EGF, and change of a myocardial cell after 24 hours was investigated morphologically and physiologically. A myocardial cell was fixed with 4% paraformaldehyde for 10 minutes, the cell area was measured using PIXEL count (Adobe Photoshop).

[0025]

As shown in Fig. 4, the cell area of the myocardial cell containing gene-transferred HB-EGF showed a significant increase (*$p<0.05$ vs Control) based on the control (HB-EGF and CD9 genes were not transferred), and the cell area of the myocardial cell containing single gene-transferred CD9 showed no significant change. On the other hand, the cell area of the myocardial cell of CD9 + HB-EGF genes transferred group

significantly suppressed increase by HB-EGF.

[0026]

Next, the beat of myocardial cells each containing gene-transferred CD9, HB-EGF, or CD9 + HB-EGF described above was recorded using a video microscope observation system (OLYMPUS microscope IX 70 + CCD camera CS 900). As a result, as shown in Fig. 5, the beat number of a myocardial cell containing gene-transferred HB-EGF showed a significant increase (130±10/min. against control 70±8/min.), however, the beat number of a myocardial cell containing gene-transferred CD9 + HB-EGF was approximately the same (76±10/min.) as the control, indicating inhibition of the action of HB-EGF by CD9 .

[0027]

A myocardial primary culture cell of a neonatal mouse was infected with Ad.CD9 at MOI 30 to cause gene transferring, CD9 was strongly expressed, and 24 hours later, human recombinant HB-EGF (rHB-EGF, R&D system, catalogue No. 259-HE)(concentration 10 ng/ml), angiotensin 2 (SIGMA, catalogue No. A-9525) (concentration 100 nM) and human recombinant HGF (rHGF, R&D system, catalogue No. 294-HG) (concentration 10 ng/ml), respectively were added into the culture solution, and allowed to act on myocardial cells for 24 hours. Thereafter, the myocardial cells were fixed with 4% paraformaldehyde, the presence or absence of transferring of CD9 was confirmed by immunostaining, and the extent of formation of muscle fiber was checked by F-actin staining. The myocardial

cell (see, photograph of Anti-CD9) containing gene-transferred CD9 shown in Fig. 6 suppressed the hypertrophy action of a myocardial cell by human recombinant HB-EGF, angiotensin 2 and human recombinant HGF, as is understood from photograph images mergeing a CD9 immunostained image and a F-actin stained image.

[0028]

Regarding myocardial cells obtained by allowing CD9 to strongly express and each adding human recombinant HB-EGF, angiotensin 2 or human recombinant HGF to the culture solution, the area ratio of the myocardial cell in each group to the area of a myocardial cell of control containing no gene-transferred CD9 was analyzed, the area of control being 1. Regarding change in beat number of a myocardial cell, Ad.CD9 or Ad.dE1.3 (control) was allowed to act sufficiently at MOI 30, from 24 hours before each addition of human recombinant HB-EGF, angiotensin 2, or human recombinant HGF. After confirmation of the presence of a beating cell, the product was washed three times with a serum-free culture solution, human recombinant HB-EGF, angiotensin 2 and human recombinant HGF, respectively were added in the same amount as in quantification of hypertrophy of a myocardial cell, and 3 hours later, 20 or more myocardial cells were observed each for 5 minutes or more using a cultured cell video observation system (OLYMPUS microscope product NO. IX 70 and CCD camera product No. CS 900), and the beat number in 1 minute was measured.

[0029]

As shown in Fig. 6, when any one of human recombinant HB-EGF, angiotensin 2 and human recombinant HGF was added, formation of muscle fiber and hypertrophy of a myocardial cell could be confirmed in all cases, however, in a myocardial cell in which CD9 had been previously strongly expressed and its expression had been confirmed, hypertrophy of a myocardial cell and formation of muscle fiber were suppressed. The cell area was statistically significant as shown in Fig. 7. For example, human recombinant HB-EGF alone: 2.8±0.5-fold to control and CD9 + human recombinant HB-EGF: 0.7±0.1-fold to control ($p<0.01$), and also in the case of angiotensin 2 and rHGF, CD9 suppressed hypertrophy of a myocardial cell likewise. The beat number of a myocardial cell showed significant increase by the action of human recombinant HB-EGF, angiotensin 2 and human recombinant HGF as shown in Fig. 8, and it was confirmed that this action is canceled by CD9 ($p<0.01$). By this, it has become apparent that CD9 suppresses a hypertrophy action of a myocardial cell and a heart positive inotropic action also in vitro.

[0030]

(Example 5)(Inhibition of phosphorylation in signal transmission)

Next, for investigating the mechanism of CD9, phosphorylation of MAPK (MAP kinase) of intracellular information transmission was carried out. From myocardial cells of which observation of beat had been completed, a protein was extracted by a Bradford method using a protein assay kit

(Biorad, catalogue No. 500-0002JA), and allowed to migrate each in an amount of 3 μg on SDS-PAGE, and recognized by Western Blotting. As a result, as shown in Fig. 9, phosphorylation of active-ERK, active-p70 S6K and active-p38 was suppressed (active-p38 was suppressed slightly) by the action of CD9 by using pERK(E-4) (SANTACRUZ catalogue No. sc-7383), p-p70 S6K(A-6) (SANTACRUZ catalogue No. sc-8416) and p-p38(D-8) (SANTACRUZ catalogue No. sc-7973), and it was guessed that signal transmission was inhibited. Inhibition of signal transmission was observed not only by the action of human recombinant HB-EGF but also by the action of angiotensin 2 and human recombinant HGF likewise, suggesting correlation to a signal transmission route of HB-EGF, angiotensin 2 and HGF. Color development was labeled with a secondary antibody of HRP, and light was emitted by super signal West Pico chemical fluorescent color developing substance (PIERCE catalogue No. 34077). As a protein of internal control, α-tublin (DM1A) (SIGMA catalogue No. T-9026) was detected by Western blotting likewise.

[0031]

(Example 6)(Improvement of cardiac function in myocardial infarction animal model)

8 to 12-week adult male mice C57/BL6 were subjected to tracheotomy, anesthetized generally (GOH nitrous oxide/oxygen/halothane) by an anesthesia machine (Kimura Medical Instrument Co., Ltd. product No. compact-15), then,

subjected to thoracotomy. A coronary artery was ligated permanently with 2-0 nylon thread, to produce myocardial infarction animal models. A PBS solution containing each 1×10^{11} particles of Ad.HB-EGF, Ad.dE1.3 and Ad.HB-EGF + Ad.CD9 dissolved in 100 ul of PBS was directly sprayed on the epicardium side of a cardiac muscle, and thoracotomy was performed. 4 days later, serum was collected, and 1 week later (1 week model after myocardial infarction) and 8 weeks later, models were tenderly sacrificed and evaluated. In the myocardial infarction 1 week model, left ventricular ejection fraction (LVEF), left ventricular diastolic diameter (LVDd), left ventricular systolic diameter (LVSd), left ventricular septum thickness (LVSt) and posterior wall thickness (PWt) were measured by echocardiography (AROKA, probe: 7.5 MHz product No. SSD-2000). From right carotid artery, an artery pressure monitor column (Millar Instruments catalogue No. SPR 407) was invasively inserted into aorta and left ventricle, and left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), left ventricular maximum positivity dP/dt and left ventricular maximum negativity dP/dt were measured (PowerLab system ver 4.2 ADInstruments). After blood collection, internal organs (heart, lung, liver, kidney, spleen) were collected.

[0032]

Fig. 10 shows a survival curve until 8 weeks in the chronic phase. Mice (HB-EGF + de1.3) containing only

gene-transferred HB-EGF died in the acute phase (within approximately 2 weeks), when CD9 was gene-transferred simultaneously, mice (HB-EGF + CD9) could survive ($p<0.05$). [0033]

Next, a histological investigation was carried out. Heart of a mouse in chronic phase 8 weeks was sliced, and fixed with 10% formalin. After paraffin embedding, the sample was cut, then, stained with HE. As shown in Fig. 11, mice containing simultaneously gene-transferred CD9 showed suppression of compensatory hypertrophy of heart as compared with mice containing gene-transferred HB-EGF. Using each section, cardiac muscle cell diameter was measured in border zone (Border: boundary region between a myocardial infarction part and a normal part) and remote zone (Remote: normal cardiac muscle part remote from a myocardial infarction part) (20 or more each samples for each zone) using LUZEX F system (manufactured by NIRECO). As shown in Fig. 12, compensatory hypertrophy of heart was observed also in the HB-EGF group in the border zone, which was also observed in the control group, and by simultaneous gene-transferring of CD9, its hypertrophy action of heart was suppressed ($p<0.05$).

[0034]

Results of measurement of the cardiac function by echocardiography and a catheter of a model one week after myocardial infarction produced for investigating a reason for 80% death of the HB-EGF + dE 1.3 group in an acute phase within

2 weeks are as described below. The cardiac function of the CD9 gene-transferred group was significantly excellent as compared with the control group and the HB-EGF gene-transferred group ($p<0.05$), in left ventricular ejection fraction (LVEF) (see, Fig. 13-1) in cardiac function evaluation by echocardiography, and in left ventricular maximum positivity dP/dt and left ventricular systolic pressure (LVSP)(Fig. 13-3 and Fig.13-4) in invasive circulation kinetics evaluation by a catheter. As told that the size of left ventricle does not change in a short period of 1 week after myocardial infarction, no difference was observed in LVDd between the CD9 gene-transferred group and the control and HB-EGF gene-transferred groups (see Fig. 13-2). Similarly, a significant difference was not observed in a short period of time of 1 week after myocardial infarction, also regarding IVSt and PWt as an index for the thickness of a wall of left ventricle (not shown). Though LVEDP showed no significant differences, there was a tendency that it was lower in the CD9 gene-transferred group (see Fig. 13-5).

[0035]

These results show that gene-transferring of CD9 into heart suppresses cardiac hypertrophy and tachycardia, and is effective for prevention and improvement of heart diseases, thus, the drug of the present invention can also be applied in other heart failure models and the like.

[Brief explanation of drawings]

[0036]

Fig. 1 shows a result of agarose electrophoresis showing expression of CD9, HB-EGF and HB-EGF receptor. H: whole heart, CM: myocardial cell, CF: cardiac fibroblast, C1; mouse lung, C2: HepG2 cell, NC: negative control, HPRT: internal control. Fig. 2 shows a graph showing a gene transferring efficiency of Ad. LacZ to a myocardial cell of a neonatal mouse, and a micrograph of a cultured myocardial cell stained with X-gal. Fig. 3 shows micrographs of myocardial cells obtained by each gene-transferring Ad. HB-EGF, Ad. CD9 or Ad. HB-EGF + Ad. CD9 to myocardial cells of a neonatal mouse and effecting immunohistochemical staining on these cells. Fig. 4 shows a graph showing areas of myocardial cells after each gene-transferring Ad. HB-EGF, Ad. CD9 or Ad. HB-EGF + Ad. CD9 to myocardial cells of a neonatal mouse. Fig. 5 shows a graph showing beat number of myocardial cells after each gene-transferring Ad. HB-EGF, Ad. CD9 or Ad. HB-EGF + Ad. CD9 to myocardial cells of a neonatal mouse. Fig. 6 shows micrographs of myocardial cells when human recombinant HB-EGF (rHB), angiotensin 2 (Ang2) or human recombinant HGF (rHGF) is allowed to act on myocardial cells of a neonatal mouse expressed by gene-transferring Ad. CD9.

Fig. 7 shows a graph showing cell areas of myocardial cells when human recombinant HB-EGF (rHB), angiotensin 2 (Ang2) or human recombinant HGF (rHGF) is allowed to act on myocardial

cells of a neonatal mouse expressed by gene-transferring Ad. CD9. In the graph, solid means gene-transferring of CD9. Fig. 8 shows a graph showing beat number of myocardial cells when human recombinant HB-EGF (rHB), angiotensin 2 (Ang2) or human recombinant HGF (rHGF) is allowed to act on myocardial cells of a neonatal mouse expressed by gene-transferring Ad. CD9. In the graph, solid means gene-transferring of CD9.

Fig. 9 shows photograph images showing analysis by Western blotting of the extent of phosphorylation in signal transmission depending on the presence or absence of expression of CD9. Fig. 10 shows a survival curve in a chronic heart failure model of adult mice 8-week after myocardial infarction. Fig. 11 shows micrographs of border zones of myocardial cells in a chronic heart failure model of adult mice 8-week after myocardial infarction. Fig. 12 shows a graph showing the diameter of a myocardial cell in border zone and remote zone in a chronic heart failure model of adult mice 8-week after myocardial infarction. Fig. 13-1 shows a graph showing LVEF of echocardiography of adult mice 1-week after myocardial infarction. In this figure, sham represents a normal mouse not revealing myocardial infarction, and MI represents myocardial infarction. Fig. 13-2 shows a graph showing LVDd of echocardiography of adult mice 1-week after myocardial infarction. Fig. 13-3 shows a graph showing + dp/dt by a catheter in adult mice 1-week after myocardial infarction. Fig. 13-4 shows a graph showing LVSP by a catheter in adult mice 1-week

after myocardial infarction. Fig. 13-5 shows a graph showing LVEDP by a catheter in adult mice 1-week after myocardial infarction.

Amended CLAIMS for the Office Action dated March 11, 2009

1. (Currently Amended) A drug for ~~preventing or~~ treating a heart disease which is associated with cardiac hypertrophy and/or tachycardia, comprising an expression vector containing a CD9 gene as the active ingredient and a pharmaceutically acceptable auxiliary or carrier.

2. (Cancelled) The drug ~~for preventing or treating a heart disease~~ according to Claim 1, wherein the heart disease is a disease causative of heart failure.

3. (Currently Amended) The drug for ~~preventing or~~ treating a ischemic heart disease, comprising an expression vector containing a CD9 gene as the active ingredient and a pharmaceutically acceptable auxiliary or carrier.

4. (Currently Amended) The drug for ~~preventing or~~ treating a ischemic heart disease according to Claim 3, wherein the ischemic heart disease is myocardial infarction.

5. (Cancelled) The drug ~~for preventing or treating a heart disease~~ according to Claim 1, wherein the heart disease includes one or more of cardiomyopathy, hypertensive heart diseases, valvular disease, congenital heart diseases and myocarditis.

6. (Cancelled) The drug ~~for preventing or treating a heart disease~~ according to Claim 1, wherein the heart disease is arrhythmia.

7. (Cancelled) The drug ~~for preventing or treating a heart disease~~ according to Claim 6, wherein the arrhythmia is tachyarrhythmia.

8. (Cancelled) The drug ~~for preventing or treating a heart disease~~ according to Claim 7, wherein the tachyarrhythmia is superventricular arrhythmia and/or ventricular arrhythmia.

9. (Cancelled) The drug ~~for preventing or treating a heart disease~~ according to Claim 6, wherein the heart disease is one or more of WPW syndrome, long QT syndrome, Brugada syndrome, and arrhythmia-inducing right ventricular dysplasia (ARVD, ARVC).

10. (Cancelled) The drug ~~for preventing or treating a heart disease~~ according to Claim 1, wherein the heart disease is associated with cardiac hypertrophy and/or tachycardia.

11. (Currently Amended) The drug for ~~preventing or~~ treating

a heart disease which is associated with cardiac hypertrophy and/or tachycardia according to Claim 1, wherein cardiac hypertrophy or tachycardia is caused by at least one of HB-EGF (heparin binding epidermal growth factor), HGF (hepatocyte growth factor) or angiotensin 2.

12. (Currently Amended) The drug for ~~preventing or~~ treating a heart disease which is associated with cardiac hypertrophy and/or tachycardia according to Claim 1, wherein the expression vector is a viral vector or a non-viral vector.

13. (Currently Amended) The drug for ~~preventing or~~ treating a heart disease which is associated with cardiac hypertrophy and/or tachycardia according to Claim 12, wherein the viral vector is adenovirus, adeno-associated virus, retrovirus, herpesvirus, herpes simplex virus, lentivirus, Sendai virus, poxvirus, poliovirus, symbis virus or vaccinia virus.

14. (Currently Amended) A method for ~~preventing or~~ treating a heart disease which is associated with cardiac hypertrophy and/or tachycardia, comprising administering an expression vector containing a sequence encoding a CD9 protein to a cardiac muscle in the heart. ~~expressing a CD9 gene in the heart of a subject in need thereof, wherein the heart disease is characterized by myocardial infarction, hypertrophy, arrhythmia, or tachyacardia.~~

15. (Cancelled) The method for ~~preventing or~~ treating a heart disease according to Claim 14, wherein the heart disease is a disease causative of heart failure.

16. (Currently Amended) The method for ~~preventing or~~ treating a ischemic heart disease, comprising administering an expression vector containing a sequence encoding a CD9 protein to a cardiac muscle in the heart.

17. (Currently Amended) The method for ~~preventing or~~ treating a ischemic heart disease according to Claim 16, wherein the ischemic heart disease is myocardial infarction.

18. (Cancelled) The method for ~~preventing or~~ treating a heart disease according to Claim 14, wherein the heart disease includes one or more of cardiomyopathy, hypertensive heart diseases, valvular disease, congenital heart diseases and myocarditis.

19. (Cancelled) The method for ~~preventing or~~ treating a heart disease according to Claim 14, wherein the heart disease is arrhythmia.

20. (Cancelled) The method for ~~preventing or~~ treating a heart disease according to Claim 19, wherein the arrhythmia is tachyarrhythmia.

21. (Cancelled) The method for ~~preventing or~~ treating a heart disease according to Claim 20, wherein the tachyarrhythmia is superventricular arrhythmia and/or ventricular arrhythmia.

22. (Cancelled) The method for ~~preventing or~~ treating a heart disease according to Claim 19, wherein the heart disease is one or more of WPW syndrome, long QT syndrome, Brugada syndrome, arrhythmia-inducing right ventricular dysplasia (ARVD, ARVC).

23. (Cancelled) The method for ~~preventing or~~ treating a heart disease according to Claim 14, wherein the heart disease is associated with cardiac hypertrophy and/or tachycardia.

24. (Currently Amended) The method for ~~preventing or~~ treating a heart disease which is associated with cardiac hypertrophy and/or tachycardia according to Claim 14, wherein cardiac hypertrophy or tachycardia is caused by at least one of HB-EGF (heparin binding epidermal growth factor), HGF (hepatocyte growth factor) or angiotensin 2.

25. (Cancelled) The method for ~~preventing or~~ treating a heart disease according to any of Claims 14 to 24, wherein the prevention or treatment is carried out by a gene therapy of transferring a CD9 gene.

26. (Cancelled) The method for ~~preventing or~~ treating a heart disease according to Claim 25, wherein the gene therapy comprises using a drug containing an expression vector containing a CD9 gene as the active ingredient.

27. (Cancelled) The method for ~~preventing or~~ treating a heart disease according to any of Claims 14 to 24, wherein the prevention or treatment comprises administration of an expression inducing substance for expressing endogenous CD9.

28. (Cancelled) The method of claim14, wherein the method comprises directly administering an adenoviral expression vector containing a sequence encoding a CD9 protein to a cardiac muscle in the heart.

29. (New) The drug for treating a ischemic heart disease according to Claim 3, wherein the expression vector is a viral vector or a non-viral vector.

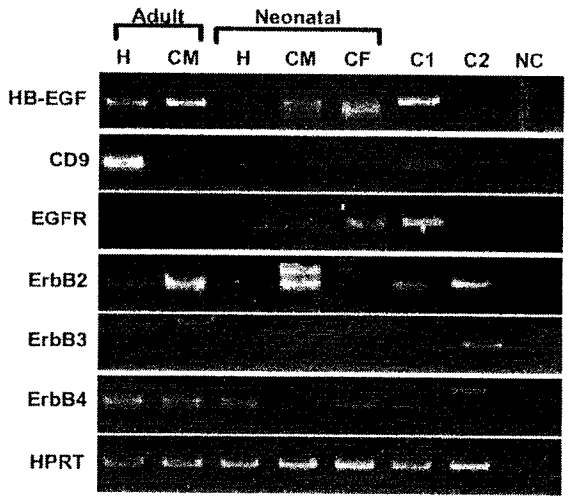
30. (New) The drug for treating a ischemic heart disease according to Claim 29, wherein the viral vector is adenovirus, adeno-associated virus, retrovirus, herpesvirus, herpes simplex virus, lentivirus, Sendai virus, poxvirus, poliovirus, symbis virus or vaccinia virus.

31. (New) The drug for treating a ischemic heart disease according to Claim 29 or 30, wherein the ischemic heart disease is myocardial infarction.

32. (New) A method for treating a heart disease which is associated with cardiac hypertrophy and/or tachycardia according to claim 14, wherein the expression vector is adenovirus.

33. (New) The method for treating a ischemic heart disease according to claim16, wherein the expression vector is adenovirus.

34. (New) The method for treating a ischemic heart disease according to Claim 33, wherein the ischemic heart disease is myocardial infarction.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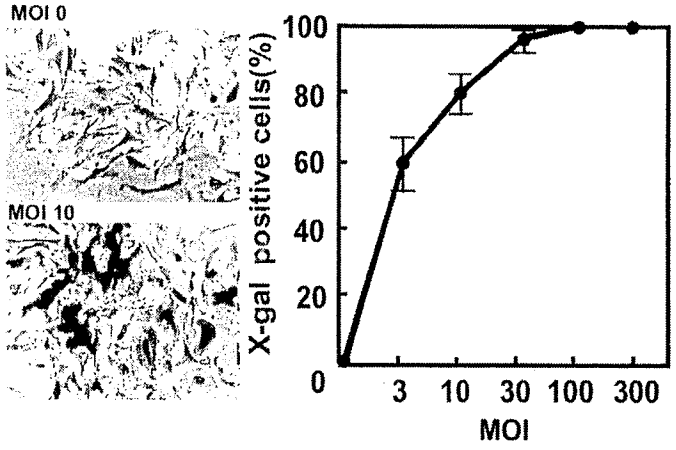
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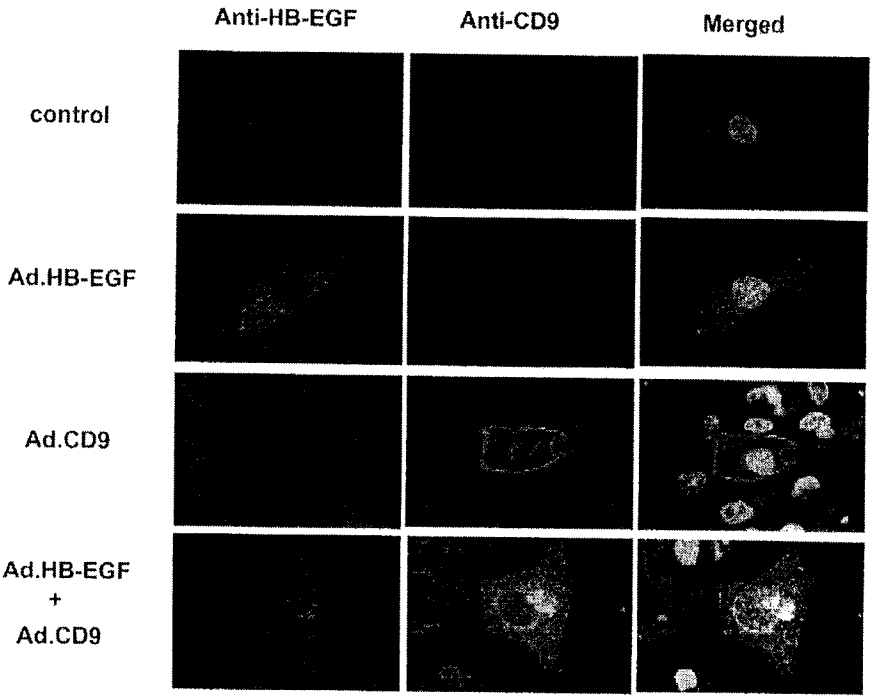
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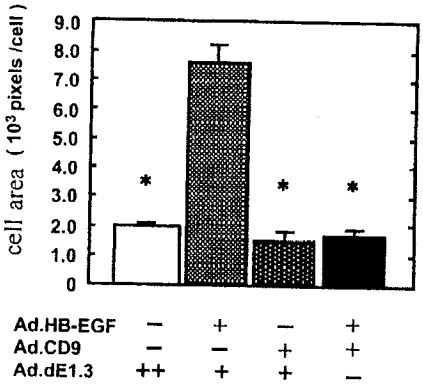
Anti-CD9
Merged
control
Ad.HB-EGF
Ad.CD9
Ad.HB-EGF
+
Ad.CD9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6.0
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*
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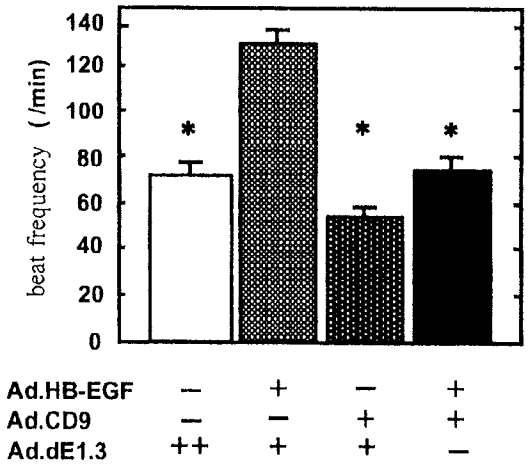
Ad.HB-EGF − + − +
Ad.CD9 − − + +
Ad.dE1.3 ++ + + −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100
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60
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20
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*
*
*
Ad.HB-EGF – + – +
Ad.CD9 – – + +
Ad.dE1.3 ++ + + –

Exhibit D

Attorney Docket No.: 55801-003US1
Client Ref. No.: PCT04TL2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	: Kenichiro Kosai *et al*	Art Unit	: 1633
Serial No.	: 10/584,109	Examiner	: Nguyen, Quang
Filed	: June 22, 2006	Conf. No.	: 8348
Title	: Drug For Preventing Or Treating Heart Diseases Comprising Cd9 Gene		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

VERIFICATION OF TRANSLATION

I, the below-named translator, hereby declare that:

My name and post office address are as stated below.

I am proficient in both the Japanese and English languages and have translated Japanese Patent Application No.: JP-2003-432279, which is in the Japanese language, into English. To the best of my knowledge and belief, this English translation, to be filed with the U.S. Patent and Trademark Office, is both true and complete.

All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of the translator: Akira Nishio

The translator's signature: A. Nishio

Date: August 07, 2009

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